Listeria Methods Development Research at the Eastern Regional Research Center, U.S. Department of Agriculture

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***Listeria* methods research at the U.S. Department of Agriculture, Eastern Regional Research Center, has concentrated on 2 areas during the past year. The first was development of techniques for assessing isolation methods for their ability to detect sublethally stressed cells. It appears that a number of widely used media do not accurately detect *Listeria* that have been injured by thermal processing or acidification. The second was development of improved plating media. One, modified Vogel-Johnson agar, shows promise; it is highly selective and quantitative, and eliminates the need to select colonies on the basis of a blue color when illuminated with reflected light.**

One attribute that distinguishes food safety microbiology and clinical microbiology is reliance of the former on quantitative methods. This reflects the need to determine both the presence and relative abundance of specific pathogens. Without this capability, it is difficult to assess the food safety importance of an isolate, particularly for species that are of public health significance only when elevated numbers are present. Therefore, when a species emerges as a potential foodborne pathogen, one of the immediate concerns is the development of effective isolation/enumeration methods that allow the quantitative detection of the microorganism from various food products.

When the Microbial Food Safety Unit at the Eastern Regional Research Center was asked to initiate research on *Listeria monocytogenes,* the priority concern identified from the literature and by virtually all of the regulatory agency and industry representatives we queried was the speed and reliability of available methodologies. Neither group could tolerate routinely analyzing samples that might require upwards of a month of cold enrichment. Product clearance requirements make this approach to isolating *Listeria* nonfeasible. As we quickly found in our laboratory, the extent of refrigerated storage space needed for maintaining samples is by itself a major logistical problem. Accordingly, methods assessment and development became one of our unit's major research thrusts in its work with *L. monocytogenes.*

After we reviewed the status of *Listeria* isolation methodology, we concentrated our initial efforts in 2 specific areas: (*1*) assessment of the ability of currently available methods to detect sublethally stressed cells, and (*2*) development of improved plating media. This presentation highlights our progress and experiences during the past year in both research areas, and gives data that we hope will be useful to laboratories that analyze foods for *L. monocytogenes.*

Sublethal Stress

Methods for the isolation of specific microorganisms from complex microflora such as would occur in foods rely on the use of selective agents. This typically involves incorporating one or more antibiotics, metal salts, dyes, or other agents or conditions that inhibit the growth of most microorganisms but do not affect the target species. The ideal selection medium would be one that supports the growth of only the target microorganism. However, most selection media are non-ideal, and often the target species is affected to some degree by the selective agents. This is particularly evident when the microorganism has been exposed to some type of sublethal stress such as heating, freezing, acidification, or drying. Direct enumeration of bacteria exposed to these conditions can lead to detecting only a small portion of the total number of cells if the target species has become sensitive to the selective agents as a result of the sublethal stress. In such instances, the microorganism must be given sufficient time in a suitable nonselective environment to facilitate repair of the damages caused by the sublethal stress. This is the rationale underlying the use of pre-enrichment steps as part of the protocol for detecting microorganisms such as *Salmonella.*

Media for the detection of *L. monocytogenes* rely heavily on the use of various selective agents, including some relatively potent antimicrobials. However, little was known about the ability of *Listeria* to maintain its resistance to these agents if pre-exposed to a sublethal stress similar to one that could occur in a food product. Failure of stressed cells to maintain resistance would result in nonquantitative recoveries because the isolation medium would detect only non-injured cells. Therefore, one of our primary goals was to assess the ability of *L. monocytogenes* isolation media to recover injured cells. We gave priority to determining the effects of sublethal heating because one of the major concerns with *Listeria* is its heat resistance.

The first step in assessing recovery of thermally injured cells is development of a method for differentiating injured and non-injured cells. Smith and Archer (1) in our laboratory and Beuchat and coworkers (2) at the University of Georgia found that the cells could be differentiated on the basis of the microorganism's ability to tolerate salt. Smith and Archer (1) observed that unheated *L. monocytogenes* (strain Scott A) grew readily on tryptose phosphate agar with either none or 5% added NaCl. However, when cultures were exposed to a mild heat process, significantly fewer cells were recovered on the 5% NaCl plates. Subsequent refinements of the system demonstrated that it is possible to heat *L. monocytogenes* at 52°C for 60 min without a decrease in viable cell numbers as determined using tryptose phosphate agar with 1% sodium pyruvate (TPAP) (Figure 1). When identical samples were plated on tryptose phosphate agar with 5% NaCl (TPAS), cell counts decreased in proportion to the duration of the heating period. This loss of tolerance is indicative of a sublethally injured cell. Accordingly, the TPAP count represents the total number of cells (injured and non-injured), the TPAS count represents the number of non-injured cells, and the number of injured *Listeria* can be calculated from the difference between the 2 counts (TPAP − TPAS).

Once a system of quantitatively differentiating injured and non-injured cells was available, it was used to assess media and media components used for the isolation of *L. monocytogenes.* Typical examples of 3 of the types of responses observed are depicted in Figure 1. These included media that could detect both injured and non-injured cells (Columbia CNA agar), non-injured cells only (LPM agar), and non-injured plus some injured cells (modified McBride agar). In addition, some media were inhibitory to non-injured cells. Media that gave recoveries substantially below that for TPAS

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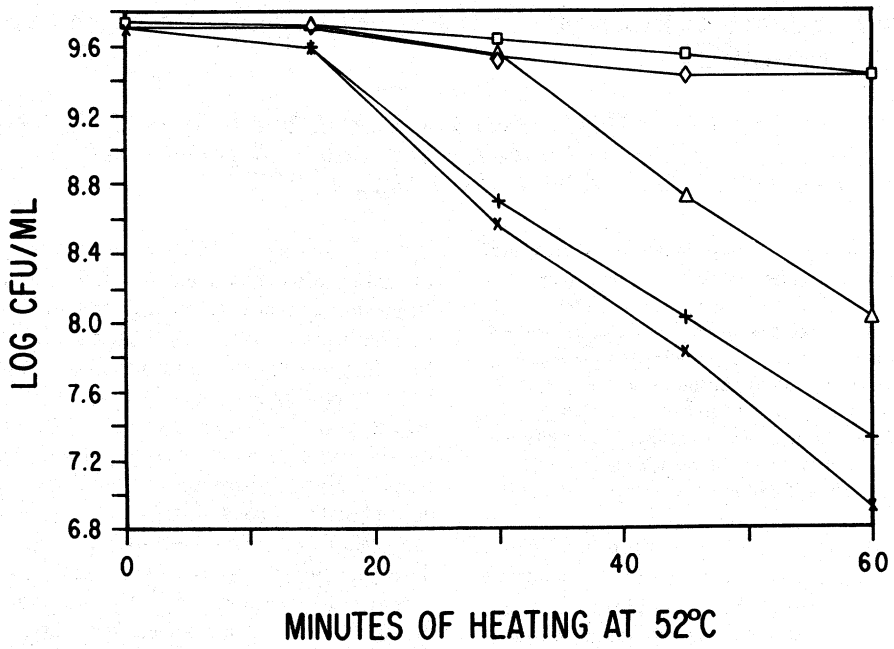

Figure 1. Colony formation by thermally injured cells of *Listeria monocytogenes*. □ = TPAP; ◇ = CNA; △ = modified McBride; + = TPAS; × = LPM.

were *Listeria* SEP broth (+2% agar) (3); brilliant green agar; GN broth, Hajna (+2% agar); MacConkey agar; thioglycollate medium; and KF *Streptococcus* agar.

A "recovery inhibition coefficient" (RIC) was developed for quantitatively comparing the various media. RIC values were calculated by plotting the data under standard conditions and then determining the differential in areas under the curves for TPAP and the test media. Using this system, the TPAP controls (injured and non-injured cells) by definition had a value of 0.0, while the TPAS controls (non-injured cells only) had a value of −51.6. The RIC values for examples of media evaluated for their ability to recover thermally stressed *L. monocytogenes* are presented in Table 1. In those cases where enrichment broth formulations were evaluated, 2% agar was incorporated to convert them to plating media.

A range of recoveries was observed among the various media; however, most of the media currently used for the isolation of *L. monocytogenes* failed to detect a substantial portion of the cells sublethally stressed by the thermal treatment. It should be emphasized that the differences in recovery rates were for injured cells only. All of these media gave approximately similar results with non-injured cells. Evaluation of these results plus additional examinations of individual medium components indicated that the inability of a medium to support colony formation by thermally injured *L. monocytogenes* could be correlated with the inclusion of the following specific compounds: phenylethanol, acriflavine, potassium tellurite, polymyxin B sulfate, sodium thiosulfate, oxgall, and sodium chloride. Initial studies have indicated that at least 6–9 h incubation in a non-inhibitory environment is needed for cells to repair the damage associated with sublethal injury, thereby regaining their resistance to NaCl and other selective agents. Work is under way in our laboratory to better define the repair process and to develop appropriate pre-enrichment protocols that will permit effective recovery of injured cells.

Thermal treatment is not the only environmental stress that can cause sublethal stress. Accordingly, our laboratory is investigating other types of injury in *L. monocytogenes*. Studies into the impact of acid injury on the recovery of *L. monocytogenes* are being completed. Initial results again indicate that a number of the media used for isolation of *L. monocytogenes* do not detect acid-injured cells. For GBNA *Listeria* medium and Columbia agar, recovery of acid-injured cells is equivalent to that for TPAP. For Vogel-Johnson agar with tellurite, phenylethanol agar, and bile esculin agar, recovery is less than that for TPAP but more than that for TPAS. Recovery is less than or the same as that for TPAS for modified McBride agar, FDA enrichment broth with 2% agar, Jay's *Listeria* medium, LPM agar, and *Listeria* enrichment broth with 2% agar.

Plating Media

Availability of suitable plating media has always been the cornerstone for performing quantitative microbiology. Although there are alternative approaches to enumeration, the isolation of individual colonies that can be examined and subsequently confirmed has always been highly desirable. Further, this approach is reasonably flexible in that, through the use of appropriate sample sizes and dilutions, direct plating can detect from relatively low numbers (approximately 10 cells/g) to high levels of a target microorganism. The major disadvantage of direct plating is typically the time required for incubation.

Isolation media for a specific bacterium are developed through the identification of suitable selection and differentiation agents or conditions. Selection is the process by which growth of nontarget species is inhibited, while differentiation assesses a characteristic or group of characteristics that provides a positive identification of the target species. Modified McBride agar (7) and LPM agar (8), two of the most widely used plating media for the detection of foodborne *Listeria*, employ combinations of antibiotics/antimicrobials for selection, and observation of blue colony color when illuminated by reflected light is the basis for differentiation. Our initial evaluation of the literature (3, 7–11) suggested 2 needs that we set as our objectives: (*1*) enhancing the selectivity of the plating media currently available, and (*2*) developing an alternative medium for direct plating that incorporates differentiation characteristics that can be determined more readily.

Table 1. Recovery inhibition coefficient (RIC) values for various media as a measure of their ability to isolate thermally injured *Listeria monocytogenes*

Medium	RIC
GBNA *Listeria* medium (4)	+1.1
TPAP (positive control)	0.0
Vogel-Johnson agar without tellurite	−5.8
Jay's *Listeria* medium (5)	−6.4
Phenylethanol agar	−13.3
Listeria enrichment broth + 2% agar (6)	−18.4
Modified McBride medium (7)	−20.2
Vogel-Johnson agar with tellurite	−28.2
Baird-Parker agar	−35.9
TPAS (negative control)	−51.6
LPM agar (8)	−62.9
FDA enrichment broth + 2% agar (7)	−64.6

Accompanying these goals we imposed additional conditions that the media be quantitative, readily obtainable, and useful to small quality-control laboratories such as those that exist throughout the food industry.

The ability of *Listeria* to reduce tellurite was chosen as an alternative means of differentiation. Tellurite has been used previously for isolation of *L. monocytogenes* (12, 13), but did not gain wide acceptance, in part due to reports that it was inhibitory (11). However, tellurite reduction is potentially a highly useful differentiation agent in that this biochemical reduction is visually distinctive and is carried out by relatively few microorganisms. Further, the compound doubles as a selective agent, suppressing the growth of a wide range of gram-negative species. Vogel-Johnson agar, a tellurite-containing medium currently used for the isolation of *Staphylococcus aureus,* supported quantitative recovery of *L. monocytogenes* from media and sterile foods. On this medium *L. monocytogenes* colonies were typically small (1–3 mm), completely black (tellurite positive), and did not produce acid (mannitol negative). Attempts to differentiate to the species level on the plating medium by incorporating xylose (only *L. monocytogenes* and *Listeria innocua* are both mannitol and xylose negative) proved unsuccessful. It appears that on this medium *Listeria* species are insufficiently fermentative to permit the use of surface plating to assess acid production from various sugars. Instead, tube cultures were needed to accurately speciate *Listeria* isolates.

Although Vogel-Johnson agar was adequate for isolating *Listeria* from samples containing only this species, when retail food samples were assayed the medium suffered significant interference due to co-contaminating micrococci, streptococci, and staphylococci. *Staphylococcus aureus* was particularly bothersome in that it occurs commonly in foods, it is tellurite positive, and its strongly positive mannitol reaction tends to mask detection of mannitol-negative *Listeria* that may also be present on the plate.

Because the interfering species were similar to those causing problems with other isolation media, subsequent work was directed toward finding agents that could be used to improve further the selectivity of both Vogel-Johnson agar and modified McBride agar. The micrococci and most streptococci proved to be controllable through the incorporation of moxalactam (Table 2) or nalidixic acid. Controlling *S. aureus* proved more difficult; it and *L. monocytogenes* had similar sensitivities to a wide range of antibiotics and other selection agents or conditions. However, it was ultimately determined that a combination of nalidixic acid, bacitracin, and moxalactam (50, 20, and 5 μg/mL, respectively) successfully suppressed *S. aureus* while permitting growth of *Listeria.* Growth was not suppressed by addition of nalidixic acid and bacitracin together or by nalidixic acid alone. This subsequently led to the formulation of ARS-modified McBride agar (ARS-MMA) and modified Vogel-Johnson agar (MVJ) (Table 3) which are currently being used and evaluated in our laboratory for detection of *L. monocytogenes* in foods.

An important characteristic of MVJ is that the tellurite reaction in *Listeria* results in colonies that are entirely black. It has been our experience with food samples that colonies on MVJ that are not entirely black prove not to be *Listeria* when subjected to confirmation. For example, mannitol-negative colonies that have a ring of white around the circumference of an otherwise tellurite-positive (black) response have consistently been found to be streptococci that are resistant to the selective agents. However, because our experience with the use of MVJ for food samples is still relatively limited,

Table 2. Effect of moxalactam on the growth of *Listeria, Staphylococcus,* and *Micrococcus* species on Vogel-Johnson agar

Microorganism	Moxalactam concentration, μg/mL					
	0	5	10	15	20	25
L. monocytogenes	+	+	+	+	+	−
L. innocua	+	+	+	−	−	−
L. ivanovii	+	−	−	−	−	−
S. aureus	+	+	+	+	+	−
Micrococcus spp.	+	−	−	−	−	−

Table 3. Formulations developed to improve selectivity of media for enumeration of *L. monocytogenes* in foods

Component	Amt used
ARS-modified McBride Agar (ARS-MMA)	
Phenylethanol agar (Difco)	35.5 g
Lithium chloride	0.5 g
Glycine anhydride	10.0 g
Cycloheximide	0.2 g
Nalidixic acid	50 mg
Moxalactam[a]	5 mg
Bacitracin[a]	20 mg
Distilled water	1000 mL
Modified Vogel-Johnson Agar (MVJ)	
Vogel-Johnson agar base (Difco)	60 g
Nalidixic acid	50 mg
Bacitracin[a]	20 mg
Moxalactam[a]	5 mg
1% Potassium tellurite solution[a]	20 mL
Distilled water	980 mL

[a] Filter-sterilized components added after autoclaving.

Table 4. Ability of ARS-MMA and MVJ to quantitatively recover *L. monocytogenes* from various foods[a]

Food	Rec.,[b] %	
	MVJ	ARS-MMA
Milk, pasteurized	102 (7)	88 (10)
Milk, sterilized	118 (19)	101 (8)
Ice cream	107 (7)	85 (6)
Cheese, Brie	147 (79)	98 (33)
Hamburger	60 (18)	60 (19)
Hamburger, irradiated	80 (27)	96 (33)
Sausage	87 (7)	94 (20)
Chicken salad	111 (17)	104 (28)
Coleslaw	105 (12)	93 (16)

[a] Foods inoculated to contain approximately 10^3 CFU/g.
[b] Values represent mean (±SEM) where n = ≥3 determinations done in duplicate.

Table 5. Use of ARS-MMA and MVJ to detect growth of *Listeria monocytogenes* Scott A in various foods after inoculation and subsequent storage for 7 days at 5°C (results as log number CFU/mL)

Food	Prior to inoculation			After inoculation			After 7 days at 5°C		
	Total mesophiles	*L. monocytogenes*		Total mesophiles	*L. monocytogenes*		Total mesophiles	*L. monocytogenes*	
		ARS-MMA	MVJ		ARS-MMA	MVJ		ARS-MMA	MVJ
Milk, pasteurized	3.14	ND	ND	3.80	3.64	3.71	5.26	5.00	5.25
Milk, sterilized	ND	ND	ND	3.82	3.83	3.98	5.46	5.02	5.21
Ice cream mix	2.16	ND	ND	3.77	3.68	3.79	5.60	4.71	5.39
Hamburger	7.84	ND	ND	7.88	3.35	3.36	8.81	3.30	3.38
Hamburger, irradiated	2.48	ND	ND	3.29	3.20	3.12	7.15	4.36	4.17
Sausage	5.88	ND	ND	5.84	3.59	3.58	7.32	3.38	3.50
Chicken salad	3.51	ND	ND	3.23	3.66	3.67	8.80	3.32	3.22
Coleslaw	4.57	ND	ND	4.70	3.59	3.65	5.05	ND	ND
Cheese, Brie	9.43	ND	ND	9.40	3.10	3.28	9.45	4.13	5.18

we subject suspect colonies that are mannitol negative and largely black to confirming tests for identification of *Listeria.*

In general, we have found MVJ to provide a slightly higher rate of recovery of *L. monocytogenes* from food samples, although both media provide recoveries that are more than adequate for quantitative purposes (Table 4). Alternatively, ARS-MMA seems to provide a higher degree of selectivity than does MVJ, although detecting *L. monocytogenes* with the latter does not suffer due to the use of tellurite as a differentiation agent. Furthermore, selectivity of both media can be enhanced by increasing the levels of antibiotics, particularly moxalactam. The antibiotic concentrations used in the formulations presented were maintained at minimal levels to ensure quantitative recovery of *Listeria.* These levels have been adequate for monitoring the growth of *L. monocytogenes* in a variety of foods (Table 5).

Currently we are assessing the performance of ARS-MMA and MVJ in comparison with LPM agar (8) for detecting the presence of *Listeria* in retail foods. Initial results indicate that both media function well, and that the additional differentiation capability of MVJ is particularly useful. This includes its use both as a direct plating medium and in conjunction with MPN enrichment techniques. The results of this assessment will be made available as soon as the study is completed.

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